Changes in the Responsiveness of Melanophores to Electrical Nervous Stimulation after Prolonged Background Adaptation in the Medaka, *Oryzias latipes*

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ABSTRACT—The effects of prolonged background adaptation on the responses of melanophores were studied using electrical nervous stimulation. Electrical stimulation at various intensities and frequencies and for various periods of time was applied to scales isolated from *B* and *W* fish and the responses of melanophores in the scales were recorded photoelectrically. When electrical stimulation at enough intensity to induce maximal melanosome-aggregation response ("maximal stimulus") was applied, there was no significant difference, in terms of the relationship between the magnitude of the aggregation response and the frequency or period of stimulation, between melanophores of *B* and *W* fish. However, the minimum effective voltage necessary to provoke the discernible aggregation of melanosomes in *B* fish was lower than that in *W* fish. With application of stimulation at intensities less than intensity of maximal stimulus, the response of melanophores in *B* fish was greater than that in *W* fish. These results suggest that prolonged background adaptation may induce changes in the excitability or in the density of distribution of chromatic nerve fibers, with a resultant change in the concentration of released norepinephrine, upon electrical stimulation.

INTRODUCTION

Color changes in teleost fish are under both neural and hormonal control, and melanophores in the skin play an important role in such changes. Bidirectional movements of melanosomes within melanophores, which are responsible for the physiological color change, are known to be regulated mainly by neural control. Norepinephrine, the neurotransmitter liberated from sympathetic postganglionic fibers, causes the aggregation of melanosomes via stimulation of α-adrenoceptors on the melanophore membrane [7, 10, 11]. In addition, various hormonal principles, such as melatonin, alpha melanophore-stimulating hormone (α-MSH) and melanin-concentrating hormone (MCH), have the ability to cause translocation of melanosomes [4, 5, 9, 15, 16]. Changes in the number and in the size of melanophores, which give rise to the morphological color change, are considered to be controlled by hormones. Several investigators have reported that the pituitary hormones, namely, α-MSH and MCH, are responsible for the morphological color change [2, 3, 22]. The two types of color change may be interrelated, but the interrelationship is not well understood.

Background adaptation is considered to be composed of two phases; an initial physiological color change and a subsequent morphological change [1, 24]. Therefore, an analysis of background adaptation may contribute to clarification of the relationship between the two types of color change. Recently, using chemically denervated medaka, Sugimoto [19] found that innervation also influenced the density of melanophores during background adaptation. Moreover, a change in the responsiveness of melanophores to exogenous norepinephrine occurred after prolonged background adaptation [19]. In the present study, therefore, we used electrical stimulation of chromatic nerves to examine, at the tissue level, whether a change in the responsiveness of melanophore to endogenous neurotransmitter liberated from sympathetic nerve fibers could be recognized after prolonged background adaptation. On the basis of our results, we discuss the effects of prolonged background adaptation on the nerves that control the aggregation of pigment in melanophores.

MATERIALS AND METHODS

Wild-type medaka, *Oryzias latipes,* of both sexes, 25–35 mm in total length, were purchased from local commercial sources.

For background adaptation, fish were maintained for 10 days in a black- or a white-background aquarium under continuous illumination at 2000 lx at the surface of the water. In the present paper, these fish are referred to as *B* fish and *W* fish, respectively. Scales isolated from the dorsal trunk of *B* or *W* fish were immersed for 10 min at 4°C in Ca^{2+}- and Mg^{2+}-free physiological saline solution (CMF-PSS) of the following composition: NaCl, 129.8 mM; KCl, 2.7 mM; D-glucose, 5.6 mM; EDTA, 1 mM; Tris-HCl buffer (pH 7.4), 5.0 mM. The epidermal layer of the scales was then ripped off with forceps, and the epidermis-free scales were kept in physiological saline solution (PSS) of the following composition: NaCl, 125.3 mM; KCl, 2.7 mM; $CaCl_2$, 1.8 mM; $MgCl_2$, 1.8 mM; D-glucose, 5.6 mM; Tris-HCl buffer (pH 7.4), 5.0 mM. These scales were used for electrophysiological studies within 3 hr of their isolation from fish.

The system employed for the perfusion of experimental solutions and the electrical stimulation of chromatic nerves was similar to that

described by Fujii & Novales [6] and Fujii & Miyashita [8].   A platinum wire of 300 μm in diameter was fully insulated, except at its tips, and used as the stimulating electrode.   The electrode was placed vertically on the central part of a scale in order to stimulate melanosome-aggregating nerves.   As an indifferent electrode, another platinum wire was dipped in the solution in the perfusion chamber at a distance of 5 mm from the stimulating electrode. Electrical stimulation at various intensities and frequencies and for various periods of time was delivered as a volley of negative pulses of 1-msec duration by means of an electric stimulator, SEN-2101 (Nihon Kohden, Tokyo).   A synchroscope, SS-5116 (Iwatsu, Tokyo) was used to monitor the stimulating pulses.

Response of each single melanophore, located at a distance of 100–200 μm from the stimulating electrode, was recorded photoelectrically.   The method used for recording the motile response of a melanophore was identical to that described by Oshima and Fujii [17].   Since there are many xanthophores in the scales of the wild-type medaka, a red filter (R-60; Toshiba, Tokyo), which blocked light with wavelengths below 600 nm, was placed across the light path in the optical part of the recording system to eliminate any influence from the motile activity of these cells.   In each series of measurements, the extent of the response of a melanophore was expressed as a percentage of the complete aggregation of melanosomes produced by norepinephrine hydrochloride at 10 μM (NE; Sankyo, Tokyo).

All measurements were performed at a room temperature, which fluctuated between 18° and 26°C.

RESULTS

After background adaptation for 10 days, which was taken as "prolonged background adaptation", remarkable differences in terms of the number and the size of melanophores were apparent between the scales of B and W fish. In both fish, electrical nervous stimulation at 5 V to central portions of isolated scales brought about the rapid aggregation of melanosomes within the melanophores (Fig. 1).   For as much as 3 hr after the isolation of scales, melanophores responded repeatedly to electrical nervous stimulation with similar aggregation of pigment.   Therefore, one and the same melanophore was observed in a series of experiments in which electrical stimulation with various parameters was applied to the scale.   The melanosomes redispersed within 4 min after cessation of electrical stimulation, and the next stimulus was applied after complete redispersion.

Effects of changes in stimulus intensity on the responses of melanophores

Figure 2 is a typical recording of melanosome-aggregating responses to electrical nervous stimulation at various intensities in a melanophore from a B fish and that from a W fish.   In these experiments, the frequency and duration were kept constant at 1 Hz and 1 msec, respectively. Stimulation was applied for 30 sec since the medaka really adapted to a white background within 30 sec.   In both B and W fish, melanin-aggregating responses started 5–10 sec after the initiation of the stimulus, and redispersion began immediately after cessation of the stimulation.   The relationship between the intensity of the stimulus and the magnitude of the response of melanophores is summarized in
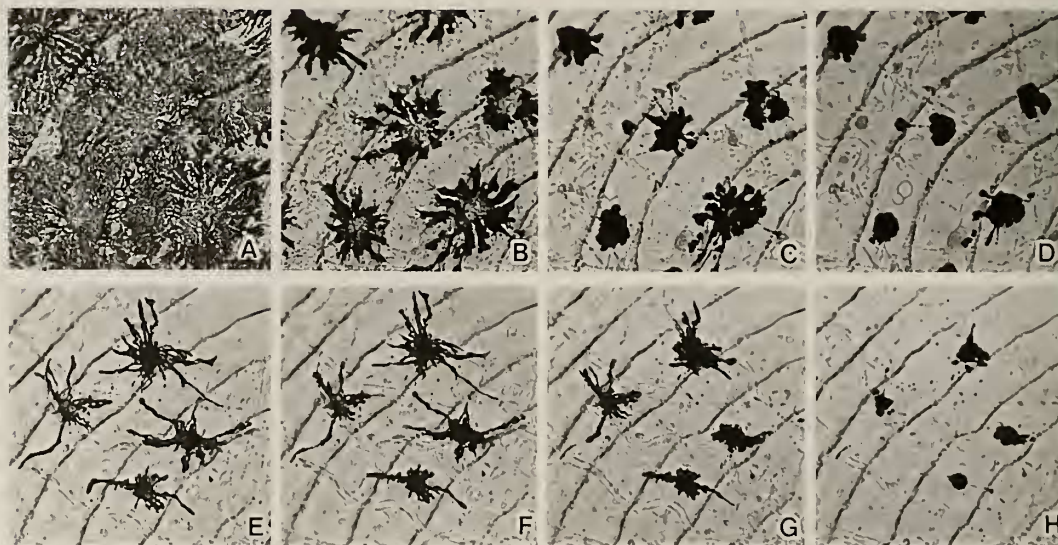

Fig. 1.   Photomicrographs showing the melanosome-aggregating responses of melanophores in scales to electrical nervous stimulation.   The upper series (A-D) shows the responses in a scale from a B fish and the lower series (E-H) shows those in a scale from a W fish.   In each series, photographs were taken with transmission optics of the same part of a given scale from the dorsal trunk.   A and E: equilibrated in PSS.   Melanosomes in melanophores are fully dispersed.   Note that larger melanophores are present at higher density in the B fish.   B and F: 15 sec after the application of stimulating pulses at an intensity of 5 V, at a frequency of 1 Hz and with a duration of 1 msec.   Melanosomes are in the process of aggregating.   C and G: 1 min after the start of electrical stimulation.   The melanosome-aggregating responses have reached the maximal level.   D and H: 4 min after treatment with 10 μNE, which was applied at the end of the electrical stimulation.   Melanosomes are completely aggregated.   Note that the extent of melanosome aggregation caused by NE is greater than that induced by the electrical stimulation (×160).
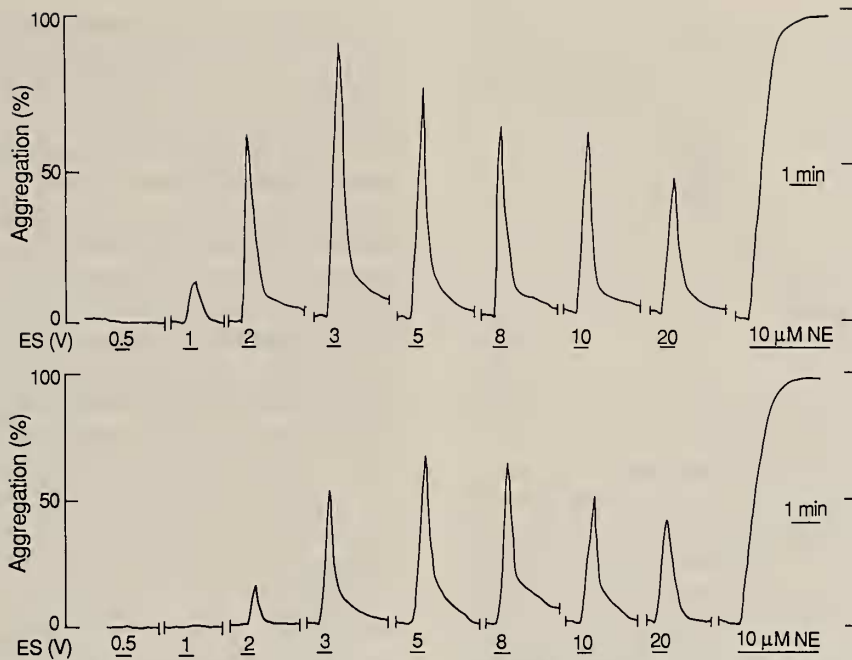
FIG. 2.   Typical recordings of the responses of melanophores to electrical stimulation at various intensities.   The upper and lower recordings show the responses of a melanophore from a *B* fish and those of a melanophore from a *W* fish, respectively.   The frequency and period of stimulation were 1 Hz and 30 sec, respectively.   After the cessation of electrical stimulation, 10 μM NE was applied for 4 min to bring about complete (100%) aggregation of melanosomes.   Although there was a difference in the threshold intensity between melanophores from *B* and *W* fish, the magnitude of the response increased depending on the intensity of the stimulus in both cases.   ES, electrical stimulation.

Figure 3.   In *B* fish, melanosomes in melanophores slightly aggregated in response to 1-V pulses and considerably in response to the intensities above 2 V.   In melanophores of *W* fish, however, no distinct aggregation response was seen at intensities of 2 V and lower.   Thus, the response of melanophores to 2-V pulses in *B* fish was significantly greater than that in *W* fish ($P<0.001$).   In melanophores of both fish, stimulation at 5 V induced the maximal response, which was about 70% of the full response caused by 10 μM NE, and a further increase in the intensity of the stimulus caused a gradual decrease in the magnitude of the response.

Effects of changes in the period of stimulation on responses of melanophores

Electrical stimulation was applied for various periods of time at an intensity of 5 V, a frequency of 1 Hz and a duration of 1 msec.   The relationship between the period of stimulation and the response of melanophores is shown in Figure 4. Five-sec stimulation, namely, five pulses of 1-msec duration over the course of 5 sec, induced discernible aggregation of melanosomes in both *B* and *W* fish.   Until the responses of melanophores reached a plateau at about 30-sec stimulation, increases in the period of stimulation were accompanied by gradual increases in the magnitude of the response.   As indicated in Figure 4, the curves for *B* and *W* fish were similar, and the response in both cases did not exceed 80% of the full response caused by NE at 10 μM.
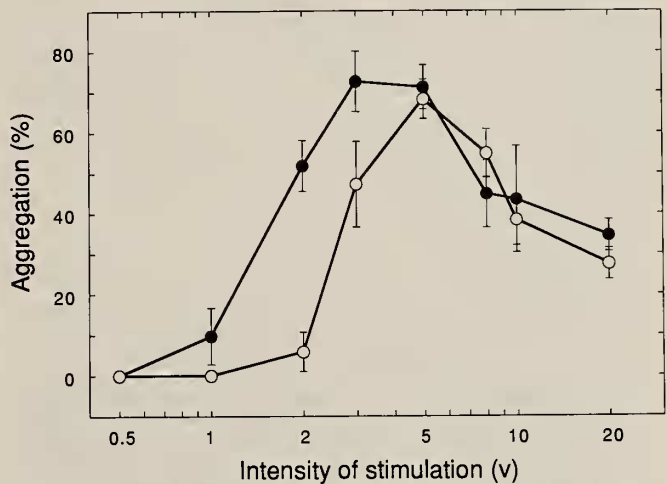

FIG. 3.   Relationship between the intensity of the stimulus and the magnitude of the melanosome-aggregating response in a single melanophore.   The curves drawn through solid and open circles indicate the relationships in *B* and *W* fish, respectively.   Stimulation was applied at a frequency of 1 Hz (1-msec duration) for 30 sec.   Complete (100%) aggregation of pigment was caused by treatment with 10 μM NE for 4 min.   Each point represents the mean of measurements on scales from five different fish.   Vertical lines indicate standard errors.

Effects of changes in the frequency of the stimulus on responses of melanophores

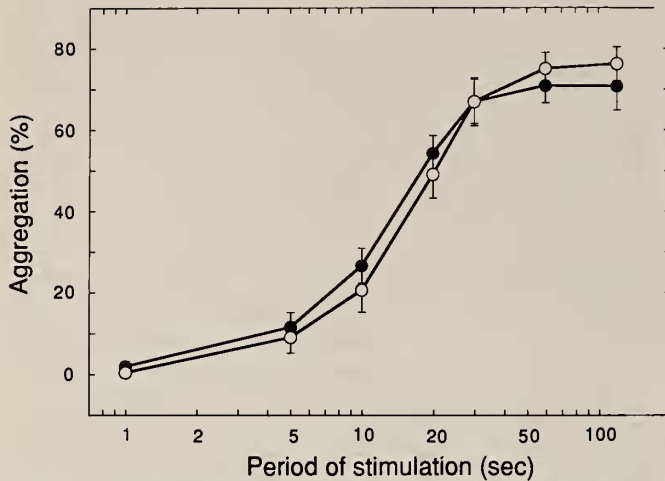The frequency of pulses also affected the responses of

Fig. 4. Relationship between the period of stimulation and the magnitude of the melanosome-aggregating response in a single melanophore. The curves through solid and open circles indicate the relationships in *B* and *W* fish, respectively. Stimulation at 5 V and 1 Hz was applied for various periods of time. Complete (100%) aggregation of pigment was caused by the treatment with 10 μM NE for 4 min. Each point represents the mean of measurements on scales from five different fish. Vertical lines indicate standard errors.
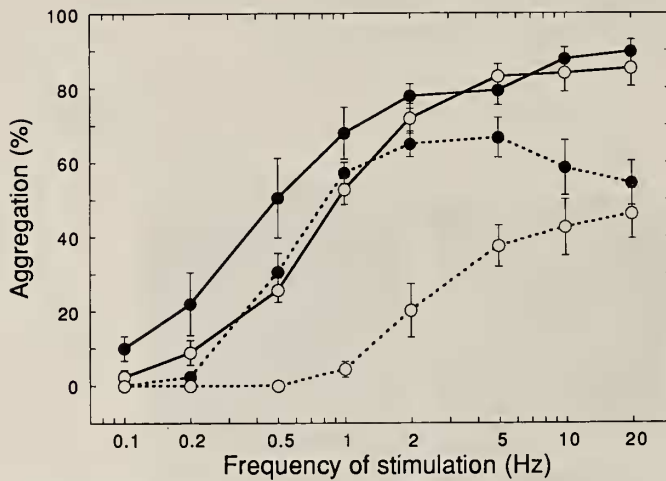

Fig. 5. Relationship between the frequency of the stimulus and the magnitude of the melanosome-aggregating reaction in a single melanophore. The curves through solid and open circles indicate the relationships in *B* and *W* fish, respectively. Stimulation was applied at an intensity of 2 V (dashed line) or 5 V (uninterrupted line) for 30 sec. Complete (100%) aggregation of pigment was caused by treatment with 10 μM NE for 4 min. Each point represents the mean of measurements on scales from five different fish. Vertical lines indicate standard errors.

melanophores. Figure 5 shows the frequency-response curves. The frequency was changed from 0.1 Hz to 20 Hz and other parameters were kept constant (5 V, 1-msec duration, 30-sec stimulation). As the frequency of the stimulation was increased from 0.1 to 5 Hz, the magnitude of the response increased by degrees. The response reached a plateau at more than 5 Hz, and the maximal responses were

about 90% of the full response to 10 μM NE in both *B* and *W* fish. There was no significant difference between the curves for *B* and *W* fish. However, frequencies below 1 Hz had a tendency to provoke a greater response in *B* fish than in *W* fish. Next, the frequency of stimulation was reduced successively without a cessation of stimulus for 4 min among measurements at each frequency, unlike the experiments mentioned above. The magnitude of the response also decreased, reaching a stable value at each frequency (Fig. 6). The effective frequencies of the stimulation for the induction of 50% aggregation of melanosomes [EF$_{50}$ (95% confidence limits)] in *B* and *W* fish, that were estimated by experimental results shown in Figure 5, were 0.66 Hz (0.22–2.00 Hz) and 1.29 Hz (0.56–2.97 Hz), respectively.
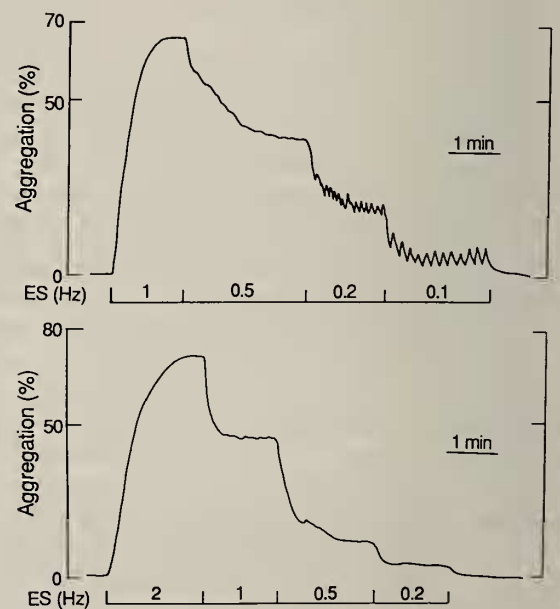

Fig. 6. Typical recordings showing the changes in the magnitude of the melanophore response with changes in the frequency. The upper panel shows a recording from a *B* fish melanophore at frequencies of 1, 0.5, 0.2 and 0.1 Hz, in turn. The lower panel shows a recording from a *W* fish melanophore at frequencies of 2, 1, 0.5 and 0.2 Hz, in turn. The intensity of the stimulus was 5 V and the duration was 1 msec. Complete (100%) aggregation of pigment was caused by treatment with 10 μM NE for 4 min. ES, electrical stimulation.

When a volley of pulses at 2 V was applied, which induced only a small response at 1 Hz in *W* fish (see Fig. 3), the threshold frequency for the induction of the response in *B* fish was lower than that in *W* fish (dashed line in Fig. 5). Within the range from 0.5 to 5 Hz, the magnitude of the response in *B* fish was significantly different from that in *W* fish ($P < 0.01$) and EF$_{50}$ (95% confidence limits) was 1.23 Hz (0.36–4.22 Hz) and 17.67 Hz (4.5–68.18 Hz), respectively. The response of melanophores in *B* fish reached a plateau value at 5 Hz, whereas that in *W* fish gradually increased with increases in the frequency. The maximal responses caused by 2-V pulses were 74% and 51% of the maximal aggregation

at 5 V in *B* and *W* fish, respectively.

DISCUSSION

Since the melanophore itself is a nonexcitable type of cell [5], neural control of its motility has been effectively studied by electrical nervous stimulation [6, 7, 8, 11, 18, 21]. Fujii and Novales [6], using split tail-fin preparations of *Fundulus*, showed that even a single stimulating pulse to chromatic nerves causes moderate aggregation of melanosomes in a melanophore. They also suggested that multiple chromatic nerve fibers might control the motility of a single melanophore since the melanosome-aggregation response increased with increases in the intensity of electrical stimulation. In the present study, we used melanophores in scales isolated from the medaka and obtained similar results for the relationship between the extent of the response and the intensity of the stimulus (see Fig. 3). More than two nerve fibers seem to be present in the scale, as well as in the tail fin. Our results are also consistent with morphological observations on the innervation of teleost melanophores reported by Yamada *et al.* [25] and Miyata & Yamada [14], who found networks of adrenergic varicose fibers labeled with [³H]-NE in the scale and the tail fin, respectively.

In general, an indication of the excitability of nerve fibers is proved by the extent of effector responses caused by stimulation at various intensities [23]. We compared responses of melanophore from *B* fish to electrical stimulation at various intensities with those of *W* fish. At 5 V, all chromatic fibers in the scales of *W* fish seemed to participate in the response because stimulation at this intensity elicited the maximal response. In *B* fish, by contrast, the maximal response was induced at 3 V, and 5-V stimulation did not further augment the magnitude of the response, which was not the full response, being only 70% of the complete aggregation of pigment caused by 10 μM NE. These findings suggest a change in the excitability of chromatic nerve fibers in response to the electrical stimulation. These data may also suggest that the density of distribution of chromatic nerve fibers in the scales of *B* fish might be higher than that in the scales of *W* fish. It is unclear why the magnitude of the response was reduced at intensities above 8 V in both fish but polarization of stimulating electrode might be involved in this phenomenon, at least to some extent.

The magnitude of the response also depended on the period of stimulation (see Fig. 4). In both *B* and *W* fish, however, the period of stimulation required for the maximal response was the same. Melanophores in scales from *B* fish possessed many thick dendritic processes, and dendrites of *W* fish melanophores were thin and small in number (see Fig. 1). Therefore, the area occupied by each melanophore of *B* fish was larger than that of *W* fish [19]. However, melanophores of *W* fish seemed to be roughly equal to these of *B* fish in the total length of a cell: the distance between the tips of two dendrites which project in an opposite direction each other with the cell body between. Thus, the distances that melanosomes would migrate within melanophores of both *B* and *W* fish might be almost the same. About 30 sec were required to induce the maximal response by stimulation at 5 V and 1 Hz.

In order to investigate the effect of prolonged background adaptation on the mechanism of release of the neurotransmitter, melanophore responses of *B* and *W* fish to various frequencies of stimulation were examined. Stimulation at 5 V was applied for 30 sec to activate all chromatic fibers and to achieve the maximal response at a given frequency. It is generally known that, when electrical stimulation at an intensity that activates all fibers is used to stimulate the peripheral neuroeffector system, the amount of NE released per stimulating pulse is constant. An increase in frequency increases the concentration of the released neurotransmitter that diffuses in the synaptic cleft [23]. Thus, the frequency-response curves in the present study indicate the relationships between the amount of NE released and the extent of the melanosome-aggregating response (see Fig. 5). Since there was no significant difference between the curves for *B* and *W* fish at an intensity of 5 V, no difference in the sensitivity of melanophores to the endogenous neurotransmitter was demonstrated. However, Sugimoto [20] reported that, as judged from the EC_{50}, the responsiveness of melanophores to exogenous NE in *B* fish was about 16 times higher than that in *W* fish. Such disagreement with the present result implies that the action of neurotransmitter released *in situ* from chromatic nerves differs from that of exogenous NE *in vitro*. In the former case, released NE is rapidly inactivated by neuronal re-uptake and metabolizing enzymes [13]. Moreover, Kumazawa and Fujii [12] demonstrated the concomitant release of ATP together with the true transmitter, NE, and they suggested that ATP is successively dephosphorylated to adenosine and functions as a cotransmitter to promote the effective redispersion of pigment. An increase in levels of released NE by high-frequency stimulation is also accompanied by increased release of ATP, with resultant prompt recovery from the effects of NE. In fact, as shown in the Figure 6, the extent of melanosome aggregation decreased quickly upon reduction of the frequency of stimulation, whereas a decrease in the concentration of exogenous NE did not rapidly reduce the extent of the responses of melanophores in the reaction chamber (data not shown). Thus, the change in the sensitivity of melanophores to NE, which was observed by Sugimoto [20] of the addition of exogenous NE, was not clear in the present experiments at the tissue level.

When electrical stimulation at 2 V was applied, the EF_{50} in *B* fish was about 14 times lower than that in *W* fish, suggesting the enhanced excitability of the chromatic fibers of *B* fish at various frequencies of electrical stimulation. The response in *B* fish reached a plateau at a frequency of 5 Hz, but the aggregation of pigment was far from complete. Probably, increases in frequency above 5 Hz failed to increase the concentration of liberated transmitter.

From the present results, we conclude that the prolonged

background adaptation affects the responses of melanophores to electrical nervous stimulation *in situ,* as a result of the changes in the excitability of nerves to stimulation and/or in the density of distribution of nerve fibers. In addition, a change in the sensitivity to exogenous NE of melanophores themselves has also been pointed out [20]. It seems likely that the change in the responsiveness of melanophores contributes to adaptation to a new background color after prolonged background adaptation. As an illustration, when fish that have fully adapted to a dark background are transferred to a new background that is brighter than the previous one, the increased excitability of chromatic nerve fibers and the increased sensitivity of melanophores to melanosome-aggregating agents may be advantageous since the fish can adapt to the new background rapidly. By contrast, decreased excitability and sensitivity may be of advantage to fish that are fully adapted to a bright background. In our next report, patterns of innervation of melanophores in *B* and *W* fish will be described.

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